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Author(s): Cunliffe, M; Whiteley, AS; Newbold, L; Oliver, A; Schafer, H; Murrell, JC

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1 **Comparison of bacterioneuston and bacterioplankton dynamics during a**
 2 **phytoplankton bloom in a fjord mesocosm**

3
 4 Michael Cunliffe¹, Andrew S. Whiteley², Lindsay Newbold², Anna Oliver², Hendrik
 5 Schäfer³ & J. Colin Murrell^{1*}

6
 7 ¹Department of Biological Sciences, University of Warwick, Gibbet Hill Road,
 8 Coventry, CV4 7AL, UK.

9 ²Centre for Ecology and Hydrology, Mansfield Road, Oxford, OX1 3SR, UK.

10 ³Warwick HRI, University of Warwick, Wellesbourne, CV35 9EF, UK.

11
 12 *For correspondence. E: j.c.murrell@warwick.ac.uk

13 T: (+44) 24 7652 3553

14 F: (+44) 24 7652 3568

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19 **ABSTRACT**

20 The bacterioneuston is the community of *Bacteria* present in surface microlayers, the
21 thin surface film that forms the interface between aquatic environments and the
22 atmosphere. In this study we compared bacterial cell abundance and bacterial
23 community structure of the bacterioneuston and the bacterioplankton (from the
24 subsurface water column) during a phytoplankton bloom mesocosm experiment.
25 Bacterial cell abundance, determined by flow cytometry, followed a typical
26 bacterioplankton response to a phytoplankton bloom, with *Synechococcus* and high
27 nucleic acid (HNA) bacterial cell numbers initially falling, probably due to selective
28 protist grazing. Subsequently HNA and low nucleic acid (LNA) bacterial cells
29 increased in abundance but *Synechococcus* did not. There was no significant
30 difference between bacterioneuston and bacterioplankton cell abundances during the
31 experiment. Conversely, distinct and consistent differences between the
32 bacterioneuston and the bacterioplankton community structure were observed. This
33 was monitored simultaneously by *Bacteria* 16S rRNA gene terminal restriction
34 fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis
35 (DGGE). The conserved patterns of community structure observed in all of the
36 mesocosms indicate that the bacterioneuston is distinctive and non-random.

37

38

39 INTRODUCTION

40 Determining and understanding both spatial and temporal patterns in bacterioplankton
41 community structure is a core aim of marine microbial ecology (15). Distributions of
42 bacterioplankton over space and time can be correlated to environmental parameters
43 and subsequent links can therefore be made to ecosystem function. A broad range of
44 spatial studies made on macro- (34), meso- (20) and micro- (27) scales have shown
45 clear patterns in distribution of the bacterioplankton.

46 The sea surface microlayer is part of the air-sea interface and is generally
47 considered to be the top 1 mm or less of the ocean (26). Surface microlayers have a
48 fundamental role in regulating transport processes between the ocean and the
49 atmosphere (26) and are often referred to as the neuston (28, 31). For over 25 years it
50 has been hypothesised that the sea surface microlayer is a hydrated gelatinous layer
51 (40) that contains surface active organic compounds such as carbohydrates, proteins,
52 lipids and humic substances, in relatively high concentrations (17, 45, 48). Recently,
53 gel-like transparent expolymer particles (TEP) have been shown to be enriched in the
54 surface microlayer, supporting the concept of a gelatinous interfacial layer (46).

55 *Bacteria* present in surface microlayers or the neuston are regarded as the
56 bacterioneuston. There are relatively few studies which have directly compared the
57 community structure of the bacterioneuston with that of the cognate subsurface
58 (bacterioplankton) in the marine environment. Analysis of *Bacteria* 16S rRNA gene
59 clone libraries constructed using DNA isolated from surface microlayer and
60 subsurface water (<1 m) samples from the North Sea revealed that the bacterioneuston
61 was dominated by two operational taxonomic units which accounted for 81% of
62 clones analysed (13). Community structure profiling using denaturing gradient gel
63 electrophoresis (DGGE) of the bacterioneuston at three sites around Oahu Island in

64 the Pacific Ocean showed that the bacterioneuston forms consistent and distinct
65 community structures. Conversely, *Archaea* community structure of the same samples
66 using *Archaea* 16S rRNA gene DGGE analysis did not show the same surface
67 microlayer-specific response, indicating that *Bacteria* and *Archaea* respond to their
68 environment in fundamentally different ways in the neuston (7).

69 Other studies, have however, reported no consistent differences between the
70 bacterioneuston and the bacterioplankton. Samples collected from two separate sites
71 in the Mediterranean Sea were analysed using single strand conformation
72 polymorphism (SSCP) of *Bacteria* 16S rRNA genes (1). The authors did not report
73 any significant differences between the surface microlayer and subsurface samples
74 using this community profiling method.

75 Non-marine studies of the bacterioneuston and *Archaea* communities in
76 estuarine (10) and freshwater (5, 19) environments have also shown distinct microbial
77 community structures present in the surface microlayer compared to those in
78 subsurface water ≤ 1 m below.

79 Recurring phytoplankton blooms are a key feature of coastal waters and
80 strongly influence bacterioplankton community structure and succession (4, 14, 38).
81 Phytoplankton blooms stimulate the bacterioplankton by the release of dissolved
82 organic matter (22) or affect bacterioplankton negatively by direct competition for
83 resources (6). Bacterioplankton community structure may also be influenced by
84 grazing flagellates or viral lysis (47).

85 Mesocosm experiments have been used to study plankton ecology for many
86 decades (33). Mesocosms facilitate study of the effects of key environmental
87 parameters, such as temperature, on plankton communities and allow the succession
88 of natural plankton communities that resemble those found in the marine environment

89 (11). The enclosed water mass means that experiments can be designed which
90 manipulate physicochemical parameters to observe biological effects. Furthermore,
91 with replicated mesocosms, the data collected can be analysed with statistics
92 rigorously. In this study we monitored the dynamics of the bacterioneuston and the
93 bacterioplankton in mesocosms of fjord surface water during an artificially induced
94 phytoplankton bloom, comparing bacterial abundance and bacterial community
95 structure in the surface microlayer and subsurface water.

96

97 MATERIALS AND METHODS

98 Mesocosm set-up and sampling

99 The experiment was carried out at the Marine Biological Field Station, Espeland,
100 Norway (20 km south of Bergen) from 21 May 2008 - 1 June 2008. Twelve land-
101 based mesocosms (1.5 m diameter and 1.5 m deep) were each filled (2,474 L) with
102 pre-filtered (~300 µm) water from the Raunefjorden. The water in the mesocosms was
103 kept mixed with submerged aquarium pumps. The mesocosms were contained in three
104 larger open containers (Figure 1A) that were filled and circulated constantly with
105 pumped fjord water to maintain the mesocosms at ambient fjord temperature. The
106 twelve mesocosms were divided into two treatment groups, control and nutrient
107 amended, allowing six replicate mesocosms for each treatment. Each of the larger
108 containers held two control mesocosms and two nutrient amended mesocosms (Figure
109 1B). Addition of nitrate and phosphate according to the Redfield stoichiometry (N:P =
110 16:1) (35), as 16µM NaNO₃ and 1µM KH₂PO₄, was used to induce the phytoplankton
111 bloom at 21.00 hours on day zero.

112 Sampling took place every day for eleven days at 09.00 hours. Subsurface
113 waters were sampled from a depth of 0.75 m in the centre of the mesocosms using a
114 siphon. The surface microlayer was sampled using two different methods, a mesh
115 screen (Garrett screen) and polycarbonate membranes taken from the centre of the
116 mesocosms. The methods sample two different depths, the mesh screen removes the
117 top ~400 µm and the polycarbonate membrane removes the top ~40 µm of the surface
118 microlayer (7). The mesh screen (16-mesh stainless steel screen: size 275 × 275 mm)
119 was placed below the surface water, lifted horizontally through the surface microlayer
120 and the water was collected into a sterile bottle. 250 mL was then filtered using a
121 peristaltic pump through a Sterivex™-GS filter unit (pore size 0.2 µm; Millipore).

122 After all the water had been evacuated from the filter unit, 1.6 mL RNAlater[®]
123 (Ambion) was added and the filter unit was stored at 4°C. Polycarbonate membranes
124 (47 mm diameter; pore size 0.2 µm; ISOPORE[™]; Millipore) were placed onto the
125 water surface using forceps and left for 10 sec before being removed and stored in 2
126 mL screw cap tubes at -20°C.

127

128 **Dissolved inorganic nutrients**

129 Subsurface water samples were filtered (Sterivex[™]-GS; pore size 0.2 µm; Millipore)
130 before being stored in polyethylene vials at -20°C until nitrate, nitrite, phosphate and
131 silicate were determined using standard segmented flow analysis with photometric
132 procedures (18).

133

134 **Phytoplankton and bacterial cell counts**

135 Phytoplankton and bacterial cells in the mesocosms were enumerated with a Becton
136 Dickinson FACScalibur benchtop flow cytometer (BD Bioscience) equipped with a
137 488 nm laser line. Cells were enumerated in samples collected from the subsurface
138 and mesh screens only, since membrane collected samples do not remove enough
139 water for flow cytometry analysis. Two analyses were performed per sample to
140 determine both phytoplankton and bacterial cell counts. Briefly, phytoplankton
141 (picoeukaryotes, coccolithophorids, small and large nanoplankton) and
142 *Synechococcus* cell counts were enumerated on fresh unstained samples using
143 modified flow rates (ca. 100 µL min⁻¹) and pre- and post- aspiration sample weighing
144 together with timed acquisition (5 min) (42). Bacterial cell counts (total count and
145 sub-sets for high nucleic acid (HNA) and low nucleic acid (LNA) bacterial cells) were
146 determined on paraformaldehyde fixed/citrate treated samples stained with SYBR[®]

147 Green I (Invitrogen) using timed acquisition (2 min) in concert with pre- and post-
148 aspiration weighing (50). For pre- and post-aspiration weighing, all samples were
149 weighed before and after analysis to determine sample volumes aspirated during the
150 sample analysis and internal 0.49 μm reference beads were used to account for flow
151 and machine drift. All analysed samples were exported as listmode files and analysed
152 using Cyflogic to gate major populations and calculate absolute cell concentrations
153 from aspirated volumes.

154

155 **Extraction of DNA for bacterial community structure analysis**

156 DNA was extracted from subsurface, mesh screen and membrane samples collected
157 on day two, day five and day ten. DNA was extracted from three control mesocosms
158 (replicates A, E and K) and three nutrient amended mesocosms (replicates B, F and L)
159 (Figure 1B). DNA was extracted in a sucrose buffer using lysozyme, proteinase K,
160 SDS and phenol-chloroform as described by Cunliffe *et al* (2008). The resuspended
161 DNA was quantified using a spectrophotometer (ND-1000; NanoDrop™) before all
162 DNA samples were diluted to a concentration of 30 $\text{ng}\cdot\mu\text{l}^{-1}$ and stored at -20°C .

163

164 **Bacterial community structure analysis**

165 PCR amplification of *Bacteria* 16S rRNA genes for T-RFLP analysis was performed
166 using a fluorescently labelled primer (6FAM)27F (5'-AGA GTT TGA TCM TGG
167 CTC AG-3') and primer 536R (5'-GWA TTA CCG CGG CKG CTG-3') (41). For
168 PCR, a total volume of 50 μl contained 0.5mM dNTPs, 0.5 μM of each primer, 2 units
169 of *Taq* DNA polymerase (Sigma) and 30 ng template DNA. The PCR programme
170 consisted of initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 1
171 min, annealing temperature 52°C for 1 min and elongation at 72°C for 3 min and then

172 a final elongation step at 72°C for 10 min. PCR products were verified by agarose gel
173 electrophoresis and stored at -20°C.

174 PCR products were purified using QIAquick PCR Purification Kit (Qiagen)
175 according to the manufacturer's instructions. 20 µl of purified PCR product was
176 digested for 4 hr at 37°C using the restriction enzyme MspI (Promega). 0.5 µl of the
177 digestion product was combined with denatured 0.5 µl LIZ600 size standard (Applied
178 Biosystems) and formamide before being run on a 3730 DNA sequencer (Applied
179 Biosystems). The sizes of the restriction fragments (T-RFs) were calculated and
180 binned using Genemarker™ (Softgenetics®). Bin widths were checked and manually
181 adjusted to encompass all concordant peaks. To differentiate signal from background,
182 a fluorescence unit threshold of 40 units was used to determine which T-RFs to
183 include. Relative abundance was calculated for each T-RF by dividing individual T-
184 RF fluorescence by total sample fluorescence.

185 PCR amplification of 16S rRNA genes from *Bacteria* for DGGE analysis was
186 performed using primers 341F (5'- CCT ACG GGA GGC AGC AG -3') and primer
187 518R (5'- ATT ACC GCG GCT GCT GG -3') (30). The same PCR was set up as
188 before for T-RFLP but using the different primers. The PCR programme for DGGE
189 consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 95°C
190 for 1 min, annealing temperature 65-55°C for 20 cycles (reduction of -0.5°C per
191 cycle) and 55°C for 15 cycles, elongation at 72°C for 1 min and then a final
192 elongation step at 72°C for 10 min.

193 DGGE was performed with a DCode™ system (Biorad). Gels were prepared
194 with 10% (v/v) acrylamide/bisacrylamide with a 30-70% linear denaturant gradient
195 (100% denaturant solution contains 6.9M urea and 11.5M formamide). The gel was
196 run in 1 × TAE buffer at 60°C for a total of 1,008 Volt hours (constant voltage 63 V,

197 16 hr). Gels were stained with SYBR[®] Gold nucleic acid stain (Invitrogen) before the
198 image was captured on a UV trans-illuminator (Syngene).

199 DGGE bands that were relatively more abundant in the surface microlayer
200 samples were selected and excised. The excised bands were washed in sterile
201 molecular grade water (MGW) before being crushed in 20 µl MGW and incubated at
202 4°C for 2hr. The eluted DNA was used to re-amplify the DGGE band using the same
203 PCR primers and conditions as before. DGGE band DNA sequences were obtained
204 using the University of Warwick Molecular Biology Services Laboratory and are
205 available in GenBank (accession numbers GQ902042 to GQ902046).

206

207 **Statistical and ordination analysis**

208 Analysis of variance (ANOVA) was used to identify statistical significance in the
209 phytoplankton and bacterial cell count data ($n = 6$; $p < 0.05$). Where significant
210 differences were seen, a Tukey's test was used to compare data within a defined set.
211 Both ANOVA and Tukey's test were performed using SPSS statistical software
212 (SPSS). Principal component analysis (PCA) was used to visualise the relationships
213 between bacterial community structures from the T-RFLP data and was carried out
214 using MINITAB[®] statistical software (Minitab). PCA is used to reduce the complexity
215 of multivariant data (T-RF relative abundance) by producing new variables that
216 account for most of the variation in the original data (39). DGGE profiles of 16S
217 rRNA genes from *Bacteria* were compared using GelCompare[®] II (Applied Maths) by
218 calculating similarity coefficients using a curve based Pearson correlation, followed
219 by the construction of Unweighted Pair Group Method with Arithmetic mean
220 (UPGMA) dendrograms from the calculated similarity coefficients.

221

222 RESULTS

223 Phytoplankton abundance

224 The phytoplankton bloom succession in the mesocosms progressed generally as
225 expected based on previous experience from earlier experiments with water collected
226 from Raunefjorden (6, 29). The nitrate and phosphate added to the nutrient amended
227 mesocosms was steadily depleted and levels returned to background concentrations by
228 day nine (Figure 2). The concentration of silicate remained constant throughout the
229 experiment. Nitrite increased in the nutrient amended mesocosms to $0.19 \pm 0.01 \mu\text{M}$
230 at day five before returning to background levels by day ten (Figure 2).

231 Phytoplankton cells were divided into four groups by flow cytometry analysis:
232 picoeukaryotes, large nanoplankton, small nanoplankton and coccolithophorids (see
233 Materials and Methods). Picoeukaryote numbers increased in both control and
234 nutrient amended mesocosms at the start of the experiment (Figure 3). By day five a
235 significant increase in picoeukaryote numbers was detected in the nutrient amended
236 mesocosms compared to control mesocosms. The artificially induced picoeukaryote
237 bloom peaked on day seven with a median cell density of $\sim 2 \times 10^5 \text{ cells.mL}^{-1}$. There
238 was no detectable significant difference between picoeukaryote cell counts in the
239 surface microlayer compared to their cognate subsurface water samples.

240 Phytoplankton cells designated as large nanoplankton showed a significant
241 increase in numbers in the nutrient amended mesocosms from day five onwards
242 (Figure 3). As with picoeukaryotes, there was no significant difference between
243 numbers in the surface microlayer and subsurface water.

244 Small nanoplankton showed more variable cell counts during the time of the
245 experiment compared to picoeukaryotes and large nanoplankton (Figure 3). After day
246 six, a significant difference was detected between the counts in the nutrient amended

mesocosms compared to cell counts in control mesocosms. The bloom of small nanoplankton peaked on day seven before returning to similar cell numbers as the control mesocosms by day nine.

As with the small nanoplankton, coccolithophorid abundance appeared stochastic in contrast to the picoeukaryotes and large nanoplankton cell counts and had no distinct trend. The intra-variation between mesocosms was high for coccolithophorid counts and this subsequently affected statistical analysis. At day seven there was a significant difference between cell counts in the subsurface samples from the control and nutrient amended mesocosms. For the remainder of the experiment the coccolithophorid counts were significantly higher in the nutrient amended mesocosms. There was also some indication of weak enrichment of coccolithophorids in the surface microlayer (Figure 3).

Bacterial abundance

Flow cytometry was used to separate three bacterial cell groups: HNA bacterial cells, LNA bacterial cells and *Synechococcus* cells. The dynamics of the three groups was different during the experiment (Figure 3).

HNA bacterial cells showed a marked decrease in abundance at the start of the experiment with the rate of decrease accelerating rapidly on day three. On day five the HNA bacterial cells numbers had dropped from an initial $\sim 6 \times 10^5$ cells.mL⁻¹ to $\sim 1 \times 10^5$ cells.mL⁻¹. After day five the abundance of HNA bacterial cells began to increase in all mesocosms and a significant difference between HNA bacterial cell counts in the nutrient amended mesocosms compared to the control mesocosms for the remainder of the experiment was detected (Figure 3). At the end of the experiment HNA bacterial cell numbers reached similar levels to those at the start of the

272 experiment. There was no significant difference in HNA bacterial cell abundance
273 between surface microlayer and subsurface water samples.

274 Unlike the HNA bacterial cells, LNA bacterial cells did not show a drastic
275 drop in abundance (Figure 3). LNA bacterial cell abundance fluctuated from day zero
276 to day eight with no overall pattern. At day two and day three there was a significant
277 difference between subsurface and surface microlayer LNA bacterial cell abundance,
278 with less cells in the surface microlayer sample. LNA bacterial cell abundance
279 fluctuated until day nine when there was a significant increase in the nutrient amended
280 mesocosms, peaking at $\sim 7 \times 10^5$ cells.mL⁻¹.

281 As with the HNA bacterial cells, *Synechococcus* cell abundance declined at
282 two rates at the start of the experiment. Initially cell abundance dropped slowly up to
283 day three and then rapidly down to $\sim 4 \times 10^3$ cells.mL⁻¹ on day six (Figure 3). Unlike
284 HNA bacterial cells, *Synechococcus* cell abundance did not recover and remained low
285 for the remainder of the experiment. There were no significant differences in
286 abundance of *Synechococcus* between treatments or between surface microlayer and
287 subsurface water.

288

289 **Bacterial community structure**

290 We used two *Bacteria* 16S rRNA gene profiling methods (T-RFLP and DGGE) to
291 monitor changes in the bacterial community structures in surface microlayer and
292 subsurface water samples collected on day two, day five and day ten.

293 PCA ordination of the structures of the bacterial communities from T-RFLP
294 analysis of subsurface and surface microlayer DNA samples is shown in Figure 4. On
295 day two, the samples collected from the subsurface and from the surface microlayer
296 using the mesh screen clustered closely together relative to the surface microlayer

297 samples collected using polycarbonate membranes. As the mesocosm blooms
298 progressed, this pattern changed drastically. At day five, samples from the subsurface
299 showed a distinct cluster that was separate from the mesh screen samples. As with day
300 two, the membrane collected surface microlayer samples remain distinct from the
301 subsurface samples. Near the end of the experiment on day ten, bacterial community
302 structure in the samples collected with the mesh screen clustered with the samples
303 collected with membranes and not subsurface water samples. Ordinance analysis of
304 the T-RFLP data in this experiment showed no evidence of bacterial community
305 structural differences as a result of the induced phytoplankton bloom (Figure 4).

306 DGGE analysis of the bacterial community structures showed similar results
307 to the T-RFLP analysis. At day two, subsurface and mesh screen-collected samples
308 were similar and membrane-collected samples showed some differences (Figure 5).
309 This was less pronounced with DGGE than with T-RFLP at day 2. By day five, the
310 membrane collected-samples were distinctly different compared to mesh screen and
311 subsurface samples, forming a separate clade in the dendrogram. Also at day five,
312 some mesh screen collected-samples were different to their associated subsurface
313 samples. By day ten, both the membrane- and mesh screen collected-samples were
314 distinctly different from the subsurface samples, corroborating the results from the T-
315 RFLP analysis. As with the T-RFLP analysis, DGGE analysis confirmed that the
316 bacterial community structures were not affected by the phytoplankton bloom.

317 Five relatively dominant DGGE bands from the surface microlayer samples
318 were excised and sequenced (Figure 5). All five DGGE band DNA sequences were
319 very similar ($\geq 98\%$) to 16S rRNA gene sequences from isolated bacterial strains
320 (Table 1). DGGE bands 1 and 2 were identical to the 16S rRNA gene sequence of
321 *Dokdonia donghaensis* PRO95 (FJ627052) and *Krokinobacter genikus* Cos-13

322 (AB198086) respectively, from the Flavobacteria family Flavobacteriaceae. DGGE
323 DNA sequences 3, 4 and 5 were almost identical to two genera, *Alteromonas* and
324 *Glaciecola* of the Alteromonadaceae (Table 1).

325 **DISCUSSION**

326 **Bacterial abundance**

327 Results show that the three bacterial cell types quantified in the mesocosms responded
328 in three different ways (Figure 3). Both HNA bacterial cells and *Synechococcus* cells
329 decreased in numbers drastically at the start of the experiment. HNA bacterial cells
330 and LNA bacterial cells then increased in numbers in the phytoplankton bloom.

331 An abrupt decrease, followed by an increase in bacterioplankton cell
332 abundance is a characteristic response frequently observed during phytoplankton
333 blooms (4, 6, 29, 36). A previous *Emiliania huxleyi*-dominated mesocosm experiment
334 using Raunefjorden fjord water showed a very similar bacterial cell response (6).
335 Other mesocosm experiments at Raunefjorden also reported the same reduction in
336 *Synechococcus* cell abundance during an induced bloom (29), thus indicating that
337 *Synechococcus* are not successful under these conditions and/or are out-competed.

338 One of the principal sources of bacterial mortality in the water column is
339 protist predation, with many protists grazing selectively (32). Significantly, some
340 protists target rapidly growing and dividing bacterial cells, such as those with HNA
341 content (16, 44). Furthermore, recent evidence suggests that the concomitant drop in
342 bacterial numbers and bloom of small phytoplankton may be due to mixotrophic
343 growth of phytoplankton (49). This may therefore account for the mortality of HNA
344 bacterial cells and *Synechococcus* cells, whereas the LNA bacterial cells did not
345 appear to be affected (Figure 3).

346 In this study, cell numbers in the bacterioneuston and the bacterioplankton
347 were not significantly different, indicating that there was no enrichment of cells in the
348 surface microlayer. Surface microlayer and subsurface water samples collected from
349 two sites in the Mediterranean Sea also showed that the numbers of *Synechococcus* in

the surface microlayer were the same as those in subsurface (0.5 m) samples (23). Bacterial cell counts by flow cytometry analysis from the same samples did have low levels of enrichment in the surface microlayer yet the enrichment of cultivable bacterial cells was much more variable, with enrichment factors ranging from 0.5 to 191 (23). High numbers of cultivable bacterial cells in the surface microlayer compared to subsurface waters are often reported (1, 2, 43).

Bacterial community structure

Unlike bacterial cell abundance, bacterial community structure was consistently different in the surface microlayer compared to subsurface water. Surface microlayer samples collected using both membranes and a mesh screen showed a reproducibly distinct bacterioneuston in the mesocosms. Previous studies have characterised the marine bacterioneuston and cognate subsurface bacterioplankton in the North Sea (13), the Mediterranean Sea (1) and Pacific Ocean (7). In the North Sea and Pacific Ocean studies the bacterioneuston community structure was distinct compared to that of the bacterioplankton 1 m below the surface (7, 13). Conversely, the Mediterranean Sea study reported no consistent differences between communities (1).

The method of surface microlayer sampling is important in the study of the bacterioneuston (7). Even though the sea surface microlayer is considered the top 1 mm of the ocean, it is operationally defined by sampling depth (26). We used a mesh screen (sampling depth ~400 μm) and membranes (sampling depth ~ 40 μm) to determine bacterial community structure. Previous comparison of membrane-collected and mesh screen-collected samples from an estuarine surface microlayer showed samples collected using a mesh screen under-represent the bacterioneuston because samples also contain subsurface water, therefore “diluting” the

375 bacterioneuston sample (7). In this study, at the start of the experiment, the mesh
376 screen-collected bacterial community structures were more similar to the subsurface
377 (bacterioplankton) than to the membrane-collected samples (bacterioneuston). This
378 however changed during the experiment with mesh screen-collected samples
379 becoming more similar to the membrane-collected samples (Figures 4 and 5). This
380 indicated an enrichment effect in the surface microlayer, causing the bacterial
381 communities sampled using the mesh screen to change from bacterioplankton-like to
382 bacterioneuston-like during the experiment.

383 The proposed enrichment of the surface microlayer and bacterioneuston may
384 be due to the physical nature of the mesocosms used in this experiment. Even though
385 the mesocosms were mixed continuously they were calmer than the open fjord.
386 Examination of surface microlayer samples offshore of Barcelona showed, that under
387 calm conditions (low wind speed and cloudless skies) the enrichment of several
388 parameters in the surface microlayer, including heterotrophic *Bacteria* counts,
389 chlorophyll-*a* and suspended particle matter, increasing substantially (23), supporting
390 our observations in the mesocosms.

391 The methodological approaches used to compare the community structure of
392 the bacterioneuston and the bacterioplankton can also influence data interpretation.
393 Agogue *et al* (2005) used similarity values based upon Jaccard coefficients of SSCP
394 profiles from surface microlayer and subsurface water samples collected in the
395 Mediterranean Sea. Jaccard coefficients are absence/presence based and do not
396 consider relative abundances (21). Franklin *et al* (2005) and Cunliffe *et al* (2009) used
397 16S rRNA gene clone libraries and DGGE profiles assessed using Pearson
398 correlations, both of which take into account the relative abundances between
399 samples. In this study we also included changes in relative abundances (T-RFs and

400 DGGE bands). The increased resolution of community structure comparisons made
401 using relative abundances versus comparisons made using absence/presence data may,
402 in part, account for the conclusions of Agogue *et al* (2005).

403 In this study, bacterial community structure dynamics in each mesocosm were
404 synchronous, showing consistent patterns between replicates (Figures 4 and 5). The
405 bacterioneuston communities at two sites on either side of Oahu Island were more
406 similar to each other than to their cognate subsurface water bacterioplankton
407 communities just 0.4 m below, also indicating non-random assembly of the surface
408 microlayer community (7). Synchronicity of discrete bacterial communities, although
409 poorly understood, is very important, as concordant community dynamics suggests
410 the community structure patterns that emerge are controlled and are not random (24).
411 Therefore, if the bacterioneuston community structure is controlled by the
412 environment and is not random, as our data suggest, then the sea surface microlayer is
413 indeed an important ecological zone of the water column.

414 Five dominant DGGE bands in the surface microlayer were sequenced and
415 identified (Figure 5 and Table 1). The bands were very similar to just two families,
416 Flavobacteriaceae (Bands 1 and 2) and Alteromonadaceae (Bands 3, 4 and 5). The
417 genera *Alteromonas* and *Glaciecola* (Alteromonadales, Alteromonadaceae) were also
418 prevalent in surface microlayer samples collected from the marine end of Blyth
419 Estuary on the North East Coast of the UK (10). A previous study has also showed the
420 closely related genus *Pseudoalteromonas* (Alteromonadales,
421 Pseudoalteromonadaceae) dominated surface microlayer samples collected from the
422 North Sea, close to the coast of the UK (13).

423

424 **Bacterial cell abundance compared to community structure**

425 Bacterioplankton in the water column include both free-living and cells attached to
426 several possible surfaces including phytoplankton (25) and marine gels (3). Marine
427 gels are a significant component of the sea surface microlayer, giving it a gelatinous
428 structure (8, 40, 46). Surface microlayer samples collected from the same mesocosms
429 in this study were enriched with transparent exopolymer gel particles (9). Therefore,
430 in the sea surface microlayer more microorganisms maybe attached than free-living
431 (8). Analysis of free-living and attached bacterioplankton communities co-occurring
432 in the water column show that both temporal variability and diversity in the attached
433 community is higher than in the free-living bacterial community (37) and specific
434 attached bacterial communities can develop (12).

435 The two standard marine microbial ecology approaches used in this study,
436 flow cytometry and community profiling (T-RFLP and DGGE), inherently analyse
437 different components of the free-living and attached bacterial cell pools. We filtered
438 the water samples for DNA extraction and subsequent community profiling, therefore
439 all particles in the water sample $> 0.2 \mu\text{m}$ were analysed by T-RFLP and DGGE, both
440 free-living and attached bacterial cell pools. However, flow cytometry only counts the
441 free-living bacterial cells. This may contribute towards the observations that there are
442 no differences in bacterial cell abundance between the surface microlayer and
443 subsurface water (free-living only), yet there are distinct and consistent differences in
444 the bacterial community structures (free-living and attached). This may also be
445 responsible for the differences reported between flow cytometry bacterial cell counts
446 and bacterial colony forming unit counts in samples collected in the Mediterranean
447 Sea by Joux *et al* (2006).

448

449 **CONCLUSIONS**

450 The similar dynamics of bacterial cell numbers and community structure between
451 replicate mesocosms described in this study shows how conserved patterns can
452 emerge in bacterial systems such as the sea surface microlayer. These data indicate
453 that the bacterial community structure patterns witnessed in the sea surface microlayer
454 are determined by environmental forces and are not idiosyncratic. This has important
455 implications for marine microbiological research as it is empirical evidence that
456 supports the hypothesis that the surface ocean, particularly the sea surface microlayer,
457 is much more structured than previously thought.

458

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470

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- 630

Table 1. Sequence similarities of excised 16S rRNA gene DGGE bands in Figure 5.

Band	BLAST Match	% similarity (no. of bases)	Taxonomic grouping
1	<i>Dokdonia donghaensis</i> PRO95 (FJ627052)	100 (158)	Flavobacteria, Flavobacteriales, Flavobacteriaceae
2	<i>Krokinobacter genikus</i> Cos-13 (AB198086)	100 (158)	Flavobacteria, Flavobacteriales, Flavobacteriaceae
3	<i>Alteromonas</i> sp. BCw006 (FJ889589)	100 (163)	Gammaproteobacteria, Alteromonadales, Alteromonadaceae
4	<i>Alteromonas</i> sp. Oct07-MA-2BB-3 (GQ215064)	100 (163)	Gammaproteobacteria, Alteromonadales, Alteromonadaceae
5	<i>Glaciecola nitratireducens</i> FR1064 (AY787042)	98 (161)	Gammaproteobacteria, Alteromonadales, Alteromonadaceae

FIGURE LEGENDS

Figure 1. (A) Photograph showing the mesocosms used in this study. Twelve mesocosms were divided into three larger containers. (B) Each mesocosm was filled sequentially A to L. Control mesocosms were A, C, E, G, I and K. The phytoplankton bloom was induced in nutrient amended mesocosms B, D, F, H, J and L.

Figure 2. Dissolved inorganic nutrient concentration changes in control (□) and nutrient amended mesocosms (■). Mean value plotted ($n = 6$) with the error bar representing the standard error.

Figure 3. Changes in abundances of phytoplankton and bacterial cells in the surface microlayer (▲) and subsurface water (■). The surface microlayer was sampled using a mesh screen. The control mesocosm samples have clear symbols and the nutrient amended mesocosm samples have solid symbols. Mean value plotted ($n = 6$) with the error bar representing the standard error.

Figure 4. Ordination diagram from PCA of bacterial T-RFLP profiles. Samples were collected on day two (red), day five (blue) and day ten (green). Subsurface water (■) was collected using a siphon and the surface microlayer was sampled using two methods: a mesh screen (▲) and polycarbonate membranes (●). The control mesocosm samples have clear symbols and the nutrient amended mesocosm samples have solid symbols.

Figure 5. Bacterial DGGE profiles from day two, day five and day ten. DGGE profiles show each replicate from the subsurface water (SS) and from the surface

microlayer sampled using a mesh screen (MS) and polycarbonate membranes (PC).

Beside each DGGE profile is the associated UPGMA dendrogram showing the similarity of the lanes in the DGGE profiles. The arrows show which DGGE bands were excised and sequenced (Table 1).



B.

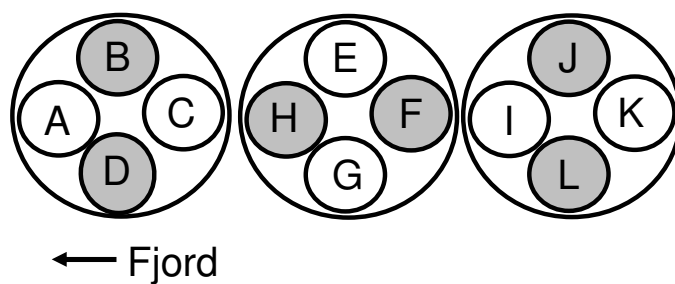


Figure 1.

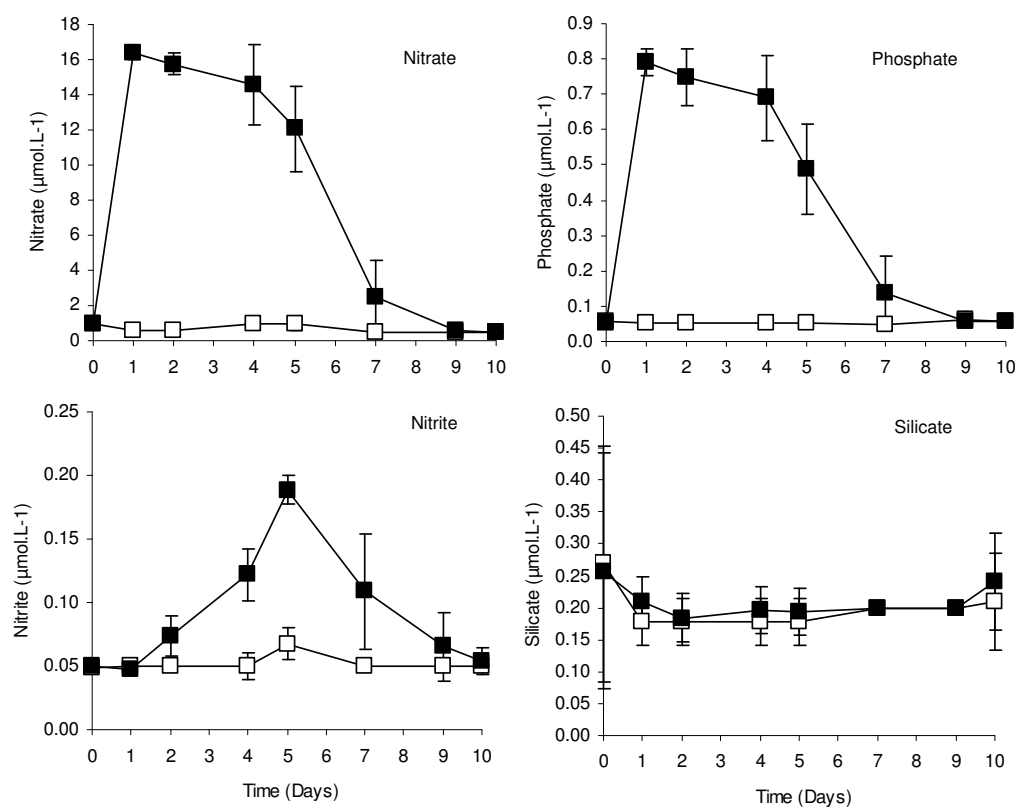


Figure 2.

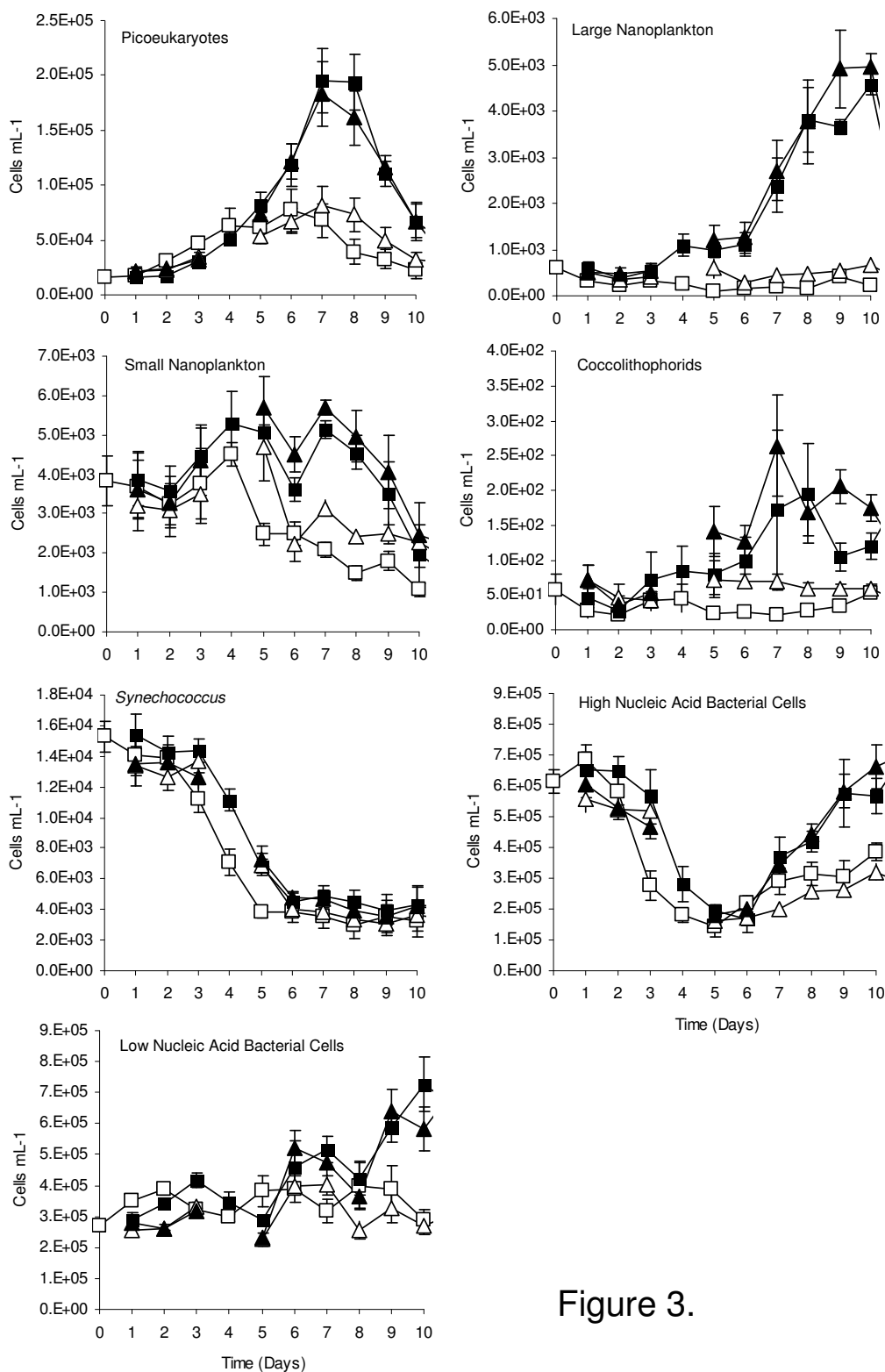


Figure 3.

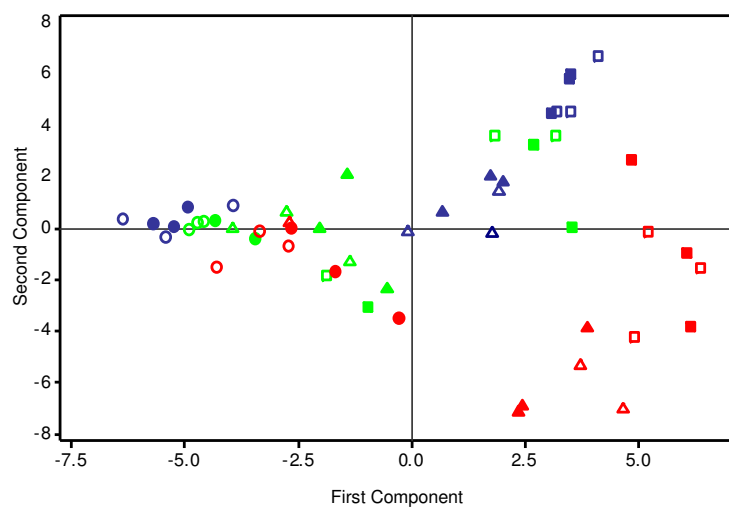


Figure 4.

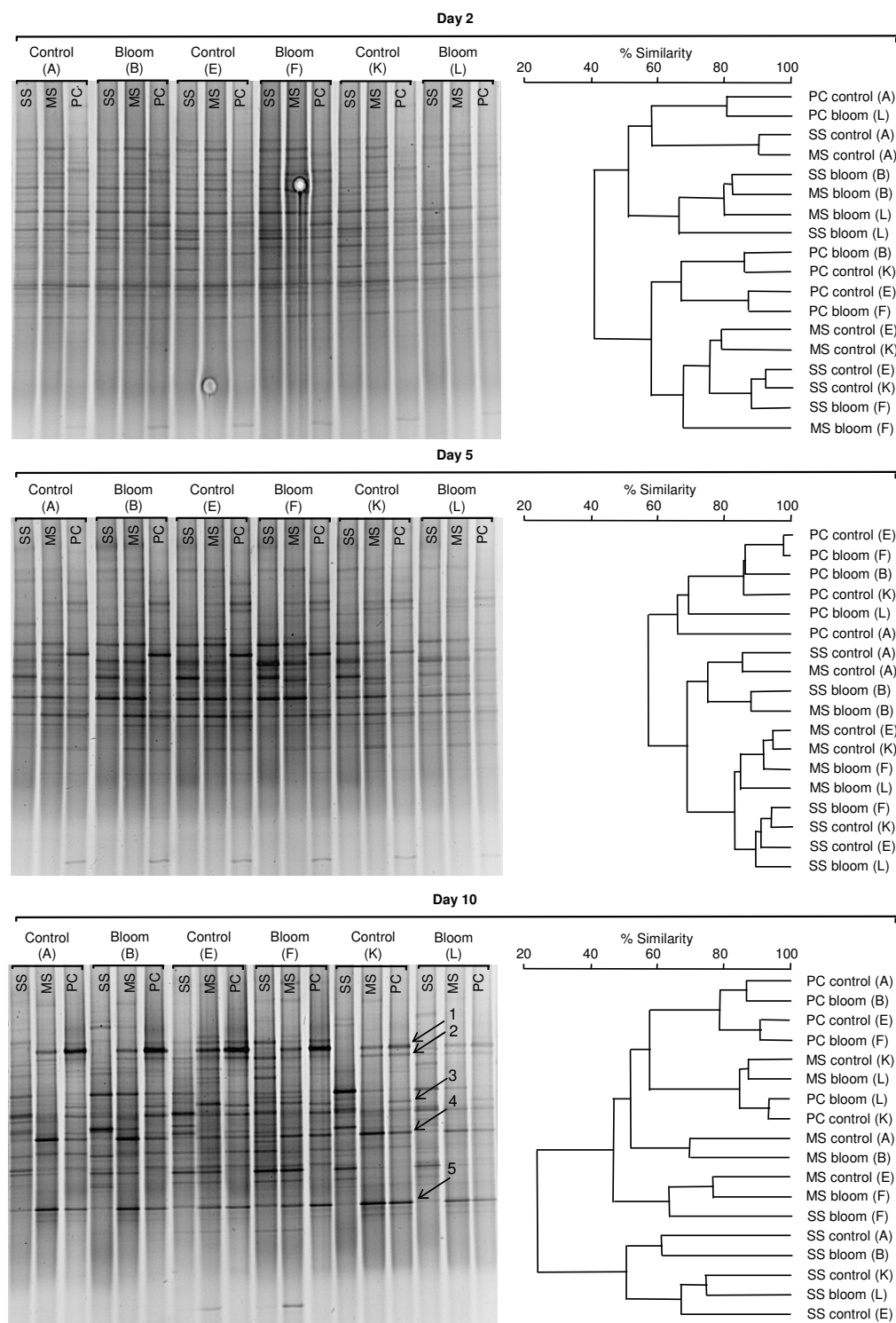


Figure 5.